REMARKS

I. Support for the Amendments

Support for claims 1-4 as amended can be found throughout the original specification as filed. More particularly, support for amended claims 1-4 can be found from page 12, line 31, to page 15, line 27, and in the Examples.

II. Status of the Claims

Claims 1-14 were originally filed with the application and were subject to a restriction requirement. Claims 1-4 (Group I) and species CYP3A4 were elected.

Claims 1-4 are currently in the application. In the Office Action dated 14 January 2003, the Examiner rejected claims 1-4 and objected to claim 2. Claims 1-4 are hereby amended.

In the Office Action Summary, the Examiner notes that claim 2 is objected to. This objection is not discussed further, and Applicants respectfully submit that they are unable to address it.

III. The Objection to the Specification Is Accommodated and Rendered Moot

The Examiner has objected to the specification on several grounds. Applicants hereby amend the specification to accommodate the Examiner's objections.

A. The Objection to the Abstract

The Examiner has objected to the Abstract. The Examiner alleges:

> The abstract of the disclosure is objected to because of poor grammar. . . . In addition, the second paragraph is a single run-on sentence. The abstract should comprise a single paragraph, not two paragraphs. Correction is required. . . . (P. 2)

Applicants hereby amend the Abstract accordingly.

B. The Objection to the Tables

The Examiner has objected to Table I, because it appears within Example 3 of the specification and again on a sheet included with the Figures. The Examiner has requested that Applicants remove the sheet of Table I included with the Figures.

Applicants hereby request that the copy of Table I on the sheet included with the Figures be removed or cancelled. Applicants hereby retain the copy of Table I within the specification text at Example 3.

C. The Objection to the Grammar

The Examiner has objected to the grammar of the specification. The Examiner alleges:

> 35 U.S.C. 112, first paragraph, requires the specification to be written in "full, clear, concise, and exact terms." The specification is replete with sentence construction that is not clear, concise and exact. The specification should be revised carefully in order to comply with 35 U.S.C. 112, first paragraph. [Examples omitted.]
>
> These two examples are not meant to comprise all problems; the applicants are required to check the specification carefully and make appropriate corrections. (Pp. 2-3.)

Applicants hereby amend the specification as outlined in the amendments and in the attached Appendix I in order to comply with the Examiner's request.

Applicants respectfully submit that the amendments to the Abstract, Table I, and the rest of the specification render the Examiner's objections moot.

IV. Rejection of Claims 1-4 Under 35 U.S.C. § 112, Second Paragraph, Is Accommodated

The Examiner has rejected claims 1-4 under 35 U.S.C., second paragraph (p. 3). The Examiner alleges:

> In Claim 1, the phrase "...capable of stably expressing..." renders the claim indefinite as, it is not clear whether the cell line does or does not express CYP1A1 etc. Claims 2-4, which are dependent on Claim 1, are rejected for the same reason.
>
> In Claims 1 and 2, for the phrase "...stably expressing...", it is unclear whether the cytochrome P450 is endogenous to the cell line or is introduced by transfection. Claims 2-4, which are dependent on Claim 1, are rejected for the same reason.
>
> For Claim 2, the interpretation of the phrase "...wherein human cytochromes P450 are capable of stably expressing CYP1A1..." is that the cytochromes P450 are expressing CYP1A1 etc. This is not correct; it is the isolated cell line that is expressing CYP1A1 etc.
>
> Appropriate corrections are required. (P. 3.)

Applicants have hereby amended claims 1-2 as provided supra. Applicants respectfully submit that the present claims 1-4 fulfill the requirements of 35 U.S.C. 112, second paragraph, and request the Examiner's reconsideration of these claims accordingly.

V. Rejection of Claims 1-2 under 35 U.S.C. § 112, First Paragraph, Is Traversed

The Examiner has rejected claims 1-2 under 35 U.S.C. 112, first paragraph (pp. 4-7). This rejection is respectfully traversed.

The Examiner alleges in part:

> Claim 1 is so broad as to encompass any human hepatic carcinoma cell line expressing any human cytochrome P450. Claim 2 is so broad as to encompass any human hepatic carcinoma cell line expressing CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP2E1. The scope of each of these claims is not commensurate with the enablement provided by the disclosure with regard to the extremely large number of cell lines broadly encompassed by the claims. Since the complement of protein expressed by a cell determines its structural and functional properties, predictability of which proteins can be changed in a cell and obtain the desired the utility of expressing any human cytochrome P450, including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP2E1, requires a knowledge of and guidance with regard to which proteins are necessary, unnecessary, or inhibitory for

the recited utility and a detailed knowledge of the ways in which each cellular protein's function relates to the function of the hepatic cell line. However, in this case the disclosure is limited to the Hepc/1A1.3, Hepc/1A2.9, Hepc/2A6L.8, Hepc/2B6.68, Hepc/2C8.46, Hepc/2C9.1, Hepc/2C19.12, Hepc/2D6.39, Hepc/2E1.3-8, and Hepc/3A4.5 cell lines.

While recombinant and screening techniques are known, it is not routine in the art to screen for alterations in multiple cellular proteins and/or multiple cell lines, as encompassed by the instant claims. Furthermore, which proteins can be altered with a reasonable expectation of success in obtaining the desired activity/utility are limited in any cell and the results of such modifications are unpredictable. In addition, one skilled in the art would expect any tolerance to alteration in expression of a single protein to diminish with each further protein altered e.g. multiple protein alterations in a single cell line.

The specification does not support the broad scope of Claims 1 and 2 which, encompasses all cell lines derived from human hepatic carcinoma that stably express any human cytochrome P450 or stably express CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP2E1. The specification does not support the broad scope of Claims 1 and 2 because the specification does not establish: (A) which proteins may be modified without effecting the ability of the hepatic cell line to stably express any cytochrome P450; (B) the general tolerance, for stable expression of any cytochrome P450 in any hepatic cell line, to modification of cellular proteins and extent of such tolerance; (C) a rational and predictable scheme for selecting hepatic cell line with modified proteins with an expectation of obtaining the desired stable expression of any cytochrome P450; and (D) the specification provides insufficient guidance as to which of the essentially infinite possible choices of hepatic cell lines with essentially infinite variations in their complement of proteins is likely to be successful.

* * *

Claims 1 and 2 are rejected under 35 U.S.C> 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

These claims are directed to a genus of human hepatic cell lines, with essentially infinite variations in their complement of proteins, which are able to stably express any cytochrome P450. The specification teaches the structure of only 14 representative species of such cell lines which, are all derived froma single parental hepatic cell line, HepG2. Moreover, the specification fails to describe any other representative species by any identifying characteristics or properties other than the functionality of being a human hepatic cell lines that is able to stably express any cytochrome P450 including CYP1A2, CYP2A6, CYP2B6, CYP2C8,

> CYP2C9, CYP2C19, CYP2D6, or CYP2E1. Given this lack of representative species encompassed by the genus of the claim, the specification fails to sufficiently describe the claimed invention in such full, clear, concise, and exact terms that a skilled artisan would recognize that applicants were in possession of the claimed invention. (Pp. 3-7.)

Applicants have amended claims 1-2 in response to the Examiner's rejection of these claims under 35 U.S.C. 112, first paragraph. Applicants respectfully submit that the present claims 1-4 fulfill the requirements of 35 U.S.C. 112, first paragraph, and request the Examiner's reconsideration of these claims accordingly.

The Examiner states that Claims 1 and 2 are too broad in view of the disclosures of the specification. Applicants respectfully disagree.

For example, cells that can be used in this invention should not be limited, for example, to HepG2 for the following reasons. HepG2 cells produce various metabolic enzymes, such as reductase, conjugation enzyme and the like, other than cytochromes P450. The cells are a cultured cell line derived from liver, in which the production of α-fetoprotein and albumin is maintained. The present invention makes it possible to evaluate metabolism totally in addition to oxidative metabolism by stable expression of cytochrome P450. Thus, it would be possible to use other liver-derived cells having properties similar to HepG2. Examples of other liver-derived cells, such as HLE, PLC/PLF/5, HuH-6, HuH-7, Hep3B, and the like, are known in the art and may be useful in the practice of the present invention. In this regard, Applicants wish to draw the Examiner's attention to Miyazaki et al., Atlas of Human Tumor Cell Lines (1994) 185-212 (copy provided with supplemental IDS submitted herewith). It is believed that one skilled in the art can practice the present invention using cell lines other than HepG2 based on the disclosure of the specification in view of the common technical knowledge as shown in the technical literature. Thus, Applicants respectfully submit that Claims 1 and 2 are well supported by the present specification.

In view of the foregoing remarks, Applicants respectfully assert that claims 1-4 fulfill the requirements of 35 U.S.C. 112, first paragraph. Therefore, Applicants respectfully request reconsideration and withdrawal of the rejections made under 35 U.S.C. 112, first paragraph.

VI. Rejection of Claims 1-3 Under 35 U.S.C. § 102(b) Is Traversed

The Examiner has rejected claims 1-3 under 35 U.S.C. 102(b) as being anticipated by Dai et al. (1993). This rejection is respectfully traversed.

The Examiner alleges:

> Claims 1-3 are rejected under 35 U.S.C. 102(b) as being anticipated by Dai et al., 1993 (in IDS). Dai et al. teach the stable expression of human P4502E1 in HepG2 cells (for example, Fig 2). Since, Claim 1 recites a cell line derived from human hepatic carcinoma capable of stably expressing a human P450, Claim 2 recites expression of CYP2E1, and Claim 3 recites HepG2 cells, Claims 1-3 are rejected under 35 U.S.C. 102(b) as being anticipated by Dai et al., 1993. (P. 7.)

Applicants respectfully submit that Dai et al. does not anticipate claims 1-3, as amended, of the present invention. Applicants have deleted the combination of HepG2 and CYP2E1 from the claims.

In view of the foregoing remarks, Applicants respectfully assert that the present invention is not anticipated by Dai et al. Therefore, Applicants respectfully request reconsideration and withdrawal of the rejections made under 35 U.S.C. 102(b).

VII. Rejection of Claims 1-4 Under 35 U.S.C. § 103(a) Is Traversed

The Examiner has rejected claims 1-4 under 35 U.S.C. 103(a) as unpatentable over Dai et al. (1993) in view of GenBank Acc# J04449 (1994) and further in view of Waxman et al. (1991). This rejection is respectfully traversed.

The Examiner alleges:

> Dai et al. do not teach stable expression of CYP3A4 in HepG2 cells. GenBank Acc#J04449 teaches the sequence of CYP3A4 (see pg 34, line 30 of the specification). It would have been obvious to a person of ordinary skill in the art to use the sequence of GenBank Acc# J04449 to prepare HepG2 cells stably expressing CYP3A4. Stably expressing CYP3A4 in HepG2 cells is suggested by Waxman et al. who teach expression of CYP3A5 in Hepc cells using vaccinia virus (Table 1) which is more difficult than using the plasmid-mediated methods of Dai et al. Motivation to use the sequence of GenBank Acc# J04449 to prepare HepG2 cells stably expressing CYP3A4 is provided by the desire to characterize the CYP3A4 enzyme in a stable, reproducible hepatic cell system. The expectation of success is high since HepG2 cells stably expressing another form P450 have been established (Dai et al., 1993). Therefore, Claims 1-4 are rejected under 35 U.S.C. 103(a) as being unpatentable over Dai et al, 1993 in view of GenBank Acc#J04449, 1994 and further in view of Waxman et al, 1991. (Pp. 7-8.)

Applicants respectfully submit that claims 1-4, as amended, of the present invention are not unpatentable over Dai et al., in view of GenBank Acc#J04449 and further in view of Waxman et al.

Dai et al. has already been discussed *supra*. As noted, Applicants have deleted the combination of HepG2 and CYP2E1 from the claims.

Because (i) Waxman et al. teach expression of CYP3A5 in Hepc cells using vaccinia virus, and (ii) the method using vaccinia virus is considered to be more difficult to perform that the plasmid-mediated method by Dai et al., the Examiner concluded that stable expression of CYP3A4 in HepG2 cells is suggested. Applicants respectfully disagree for the following reasons.

(1) Generally, a gene transfer system using vaccinia virus is usable for transient expression, which is totally different from a stable or constitutive expression system. Waxman et al. utilize CYP transiently expressed in the cell as a microsome (enzyme) to obtain CYP activity. Transient expression and stable expression are considered to be quite different expression techniques. In general, in the case of transient expression, a vaccinia virus vector remains in the cytoplasm and replicates autonomously in the cytoplasm,

expressing a target gene in the cytoplasm. In general, a high level of expression is obtained, but by passage of the cell transfected with the vaccinia virus vector, the level of expression is reduced. On the other hand, in the case of stable (or constitutive) expression, a target gene is integrated into the chromosome of the cell via homologous recombination, an intrinsic cellular mechanism. Although the expression level is not as high in comparison to the level observed for transient expression, the stable/constitutive expression is not reduced by the passage of cells. Thus, the possibility of stable expression of CYP3A4 cannot be suggested by transient expression of CYP3A4 using vaccinia virus. One of ordinary skill in the art would not assume, simply because a certain protein can be transiently expressed in a cell, that the same protein could be stably expressed in the cell.

(2) The choice of a plasmid-mediated method has a sound technical basis (i.e., to establish stable expression of cytochromes P450) and is not merely a matter of convenience. For stable expression, a target must be integrated into the chromosome of the cell (as discussed *supra*). Because a homologous recombination mechanism is utilized for integration of the target into the chromosome, integration of multiple copies of the gene is quite difficult to achieve. Indeed, in the disclosure of Dai et al., only one copy of the viral DNA was successfully integrated into the genome. The successful establishment of a stable expression system depends on the type of cell utilized. Applicants assert that the plasmid-mediated method described by Dai et al. is more difficult to utilize than the method using a vaccinia virus. Therefore, there is no motivation in Waxman et al. or in Dai et al. to combine these two references to establish the stable expression of CYP3A4 using HepG2.

(3) In addition, the most beneficial use of a stable expression system is its use in providing a means for observing metabolic activities in living cells. By providing a means of observing metabolic activities in living cells, the following can be studied: (i) the process of metabolism by CYP of a compound of interest; (ii) any metabolic relationship between CYP and enzymes that the cell inherently possesses; (iii) a reduction of toxicity associated with the metabolism by CYP of a compound of interest; and (iv) the effects of a metabolite incurred by CYP on the cells. Other uses will occur to one of ordinary skill in the art.

Therefore, Applicants assert that stable expression using the combination of HepG2 and CYP3A4, as well as other claimed combinations, would not have been obvious to one of ordinary skill in the art at the time the invention was made.

In view of the foregoing remarks, applicants respectfully assert that the present invention would not have been obvious over Dai et al. (1993) in view of GenBank Acc# J04449 (1994) and further in view of Waxman et al. (1991). Therefore, applicants respectfully request reconsideration and withdrawal of the rejections made under 35 U.S.C. § 103(a).

VIII. Conclusion

In view of the foregoing amendments and remarks, the present application is respectfully considered in condition for allowance. An early reconsideration and notice of allowance are earnestly solicited.

It is believed that all outstanding rejections have been addressed by this submission and that all the claims are in condition for allowance. If discussion of any amendment or remark made herein would advance this important case to allowance, the Examiner is invited to call the undersigned as soon as convenient.

Applicants hereby request a two-month extension of time for the Amendment and accompanying materials. Although it is not believed that any additional fee (in addition to the fee concurrently submitted) is required to consider this submission, the Commissioner is hereby authorized to charge our deposit account no. 04-1105 should any fee be deemed necessary.

Respectfully submitted,

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APPENDIX I

REVISIONS OF THE SPECIFICATION PURSUANT TO REVISED RULE § 1.121

In the Specification:

Please replace the paragraph from page 2, line 29, to page 4, line 26, with the following paragraph:

-- Hepatocytes are known to have a great many physiological functions, all of which ~~and of all those,~~ play a very important function in terms of the metabolism of xenobiotics and/or endogenous substrates such as drugs, food additives, environmental pollutants, industrial chemicals and the like. At the same time, the function of metabolizing xenobiotics and/or endogenous substrates might lead to ~~induce~~ inducing the inhibition of metabolizing enzymes for xenobiotics and/or endogenous substrates by xenobiotics and/or endogenous substrates, to accelerate the activity of metabolizing enzymes for xenobiotics and/or endogenous substrates, to express cytotoxicity by the metabolism of xenobiotics and/or endogenous substrates, to express genotoxicity by the metabolism of xenobiotics and/or endogenous substrates, to express carcinogenicity by the metabolism of xenobiotics and/or endogenous substrates, to express mutagenicity by the metabolism of xenobiotics and/or endogenous substrates, to express hepatotoxicity by the metabolism of xenobiotics and/or endogenous substrates, and so on. For these reasons, the function of xenobiotics and/or endogenous substrates has been widely studied. It is known that many enzymes are associated with the metabolism of xenobiotics and/or endogenous substrates referred to herein. Examples of such enzymes include UDP-glucuronosyltransferase, sulfotransferase, glutathione transferase, epoxy hydratase, N-acetyltransferase, flavin monooxygenase and cytochromes P450. ~~And,~~ Also, the presence of a cytochrome P450 reductase is crucial ~~to express~~ for expressing the enzymatic function of cytochromes P450. Of an array of these enzymes, cytochromes P450 play the most important role in the metabolism of xenobiotics and/or endogenous substrates. The term cytochromes P450 collectively refers to a class of enzymes including a great many molecular species. In the metabolism of xenobiotics and/or

endogenous substrates in human liver, ten (10) species of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 are considered important. Also, these enzymes, which are distributed in human liver, are functionally different depending on species and hence, human-derived liver specimens are unable to ~~use~~ be used as a stable test system. On the other hand, such a metabolic function of the liver involves a very strong specificity, i.e., differences in nature, depending on species, which makes it difficult to predict such diverse metabolic functions of human liver from experimental animals, e.g., rats. However, it is practically impossible to analyze these ~~items~~ functions of interest in ~~human~~ humans. For these reasons, human-derived cultured hepatocytes are considered useful not only in examining the function of human liver in a rapid, inexpensive, safe and accurate way ~~provide~~ provided in place of experimental animals, but also in producing a so-called artificial liver as a functional substitute for human liver ~~in function~~. However, it is impossible to subculture ~~human~~ normal human hepatocytes separated from tissues in vivo. Cells that can be established ~~into~~ as a cell line often lack the differentiation capability possessed inherently and in most cases, do not exactly reflect the function of tissues to which the cell line originally belongs. A family of enzymes that metabolize xenobiotics and/or endogenous substrates especially in liver cells, among others, the family of cytochromes P450 molecular species loses its activity in an extremely short period of time in primary culture; any cell line that fully retains the property has not been found so far (J. Dich et al., Hepatology, 8, 39-45 (1988)). Thus, in light of the foregoing, there is an extensive need for hepatocytes that can retain the capability of metabolizing xenobiotics and/or endogenous substrates and can be incubated.--

Please replace the paragraph from page 4, line 27, to page 5, line 10, with the following paragraph:

-- To date, however, no cultured cell line has been obtained as retaining the function associated with the metabolism of xenobiotics and/or endogenous substrates as in the liver. Particularly because the activity of cytochromes P450 is widely recognized to be rapidly lost in cultured cells, it has been hitherto attempted to stably express cytochromes P450 in the established cultured cells and by this, take over the metabolizing function of liver (M. Sawada

et al., Mutation Research, 411, 19-43 (1998)). However, as stated above, the cell line ~~to express~~ for expression of cytochromes P450 should indispensably be derived from human liver cells. In addition, the activity of NADPH cytochromes P450 reductase is required for expressing the activity of cytochromes P450, requiring further expression of many more enzymes. Therefore, stable and safe reproduction of the metabolizing function in human liver should be in human-derived cultured hepatocytes that retain the activity of enzymes participating in the metabolism of cytochromes P450 as well as various other metabolisms.--

Please replace the paragraph from page 5, line 11, to page 6, line 34, with the following paragraph:

-- As examples of the expression of cytochromes P450 in cells retaining the activity of various enzymes participating in metabolism, there are cases in which P450 was expressed in HepG2 cells using vaccinia virus (Methods in Enzymology, T. Aoyama et al. in Methods in Enzymology, 260, 85-92, edited by M. R. Waterman, Academic Press, 1991) and in which CYP2E1 was expressed in HepG2 cells (Y. Dai et al., Biochemistry, 32, 6928-6937, 1993). In the former case, careful handling is required, which is an obstacle to practical application. The latter was attempted for CYP2E1 alone but so far ~~none~~ has not been attempted for many other species of cytochromes P450 present in the liver. Accordingly, if a cultured cell line that can retain the activity of a family of enzymes participating in the metabolism of xenobiotics and/or endogenous substrates in the liver could be obtained, this ~~will~~ would enable a practitioner to (1) analyze an enzyme participating in the metabolism of xenobiotics and/or endogenous substrates, (2) analyze a metabolic pathway of xenobiotics and/or endogenous substrates, (3) analyze a chemical structure of the metabolite of xenobiotics and/or endogenous substrates, (4) prepare the metabolite of xenobiotics and/or endogenous substrates, (5) analyze inhibition of the metabolizing enzyme for xenobiotics and/or endogenous substrates, (6) analyze an accelerated activity of the metabolizing enzyme for xenobiotics and/or endogenous substrates, (7) analyze expression of cytotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (8) analyze expression of genotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (9) analyze expression of carcinogenicity by the metabolism of xenobiotics and/or endogenous substrates,

(10) analyze mutagenicity by the metabolism of xenobiotics and/or endogenous substrates, (11) analyze expression of hepatotoxicity by the metabolism of xenobiotics and/or endogenous substrates, and (12) analyze xenobiotics and/or endogenous substrates that act on the liver.~~; furthermore, such will~~ Furthermore, this would enable a practitioner to (1) screen a substance capable of inhibiting xenobiotics and/or endogenous substrates, (2) screen a substance capable of accelerating the activity of metabolizing enzymes for xenobiotics and/or endogenous substrates, (3) screen a substance capable of expressing cytotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (4) screen a substance capable of expressing genotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (5) screen a substance capable of expressing carcinogenicity by the metabolism of xenobiotics and/or endogenous substrates, (6) screen a substance capable of expressing mutagenicity by the metabolism of xenobiotics and/or endogenous substrates, (7) screen a substance capable of expressing hepatotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (8) screen xenobiotics and/or endogenous substrates which act on the liver, and (9) screen a substance capable of acquiring a new physiological activity or increasing or decreasing the inherent physiological activity, through the metabolism of xenobiotics and/or endogenous substrates. Thus, specific compounds or salts thereof, etc. can be obtained using the method for analysis and/or the method for screening above.--

Please replace the paragraph from page 7, line 8, to page 8, line 17, with the following paragraph:

-- These cells enable a practitioner of the invention to (1) analyze an enzyme participating in the metabolism of xenobiotics and/or endogenous substrates, (2) analyze a metabolic pathway of xenobiotics and/or endogenous substrates, (3) analyze a chemical structure of the metabolite of xenobiotics and/or endogenous substrates, (4) prepare the metabolite of xenobiotics and/or endogenous substrates, (5) analyze inhibition of the metabolizing enzyme for xenobiotics and/or endogenous substrates, (6) analyze an accelerated activity of the metabolizing enzyme for xenobiotics and/or endogenous substrates, (7) analyze expression of cytotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (8) analyze expression of genotoxicity by the metabolism of xenobiotics and/or endogenous

substrates, (9) analyze expression of carcinogenicity by the metabolism of xenobiotics and/or endogenous substrates, (10) analyze mutagenicity by the metabolism of xenobiotics and/or endogenous substrates, (11) analyze the expression of hepatotoxicity by the metabolism of xenobiotics and/or endogenous substrates, and (12) analyze xenobiotics and/or endogenous substrates that act on the liver. The cells further enable a practitioner of the invention to (1) screen a substance capable of inhibiting xenobiotics and/or endogenous substrates, (2) screen a substance capable of accelerating the activity of metabolizing enzymes for xenobiotics and/or endogenous substrates, (3) screen a substance capable of expressing cytotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (4) screen a substance capable of expressing genotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (5) screen a substance capable of expressing carcinogenicity by the metabolism of xenobiotics and/or endogenous substrates, (6) screen a substance capable of expressing mutagenicity by the metabolism of xenobiotics and/or endogenous substrates, (7) screen a substance capable of expressing hepatotoxicity by the metabolism of xenobiotics and/or endogenous substrates, (8) screen xenobiotics and/or endogenous substrates which act on the liver, and (9) screen a substance capable of acquiring a new physiological activity or increasing or decreasing the inherent physiological activity, through the metabolism of xenobiotics and/or endogenous substrates. Thus, particular compounds or salts thereof, etc. can be obtained, using the method for analysis and/or the method for screening.--

Please replace the paragraph at page 8, lines 18-25, with the following paragraph:

-- In view of the foregoing problems, the present inventors have made extensive studies. ~~and as~~ As a result, they have established stable transformants capable of stably expressing cytochromes P450 in a human hepatocarcinoma-derived (or hepatic carcinoma-derived) cell line with an enhanced activity ~~that participate~~ for participation in the metabolism of xenobiotics and/or endogenous substrates. The following further studies have resulted in accomplishing this invention.--

Please replace the paragraph at page 13, lines 6-11, with the following paragraph:

-- The human hepatic carcinoma cells used can be collected by separating a human

hepatic carcinoma-derived cultured cell line (preferably HepG2) from human hepatic carcinoma. Genes that encode various species of cytochromes P450 separately ~~separated~~ isolated are stably expressed in the human hepatic carcinoma cells.--

Please replace the paragraph at page 13, lines 12-30, with the following paragraph:

-- In order to stably express DNA fragments encoding cytochromes P450, first, DNA fragments encoding, e.g., individual cytochromes P450 are obtained and placed under control of a foreign promoter for expression. The base sequences of DNA fragments encoding cytochromes P450 are available from public database. Based on the base sequences, a cytochromes P450-encoding DNA fragment can be ~~separated~~ isolated by publicly known methods including PCR, hybridization screening, etc. The DNA fragment thus obtained is inserted into a vector which produces transformants capable of stably expressing a foreign gene in a ~~mammal~~ cultured mammalian cell, whereby a vector for transformation is produced. The resulting vector is transfected ~~to~~ into hepatic carcinoma cells by publicly known methods. Transformants are selected by examining the enzyme activity induced by the expression of cytochromes P450 transformed therein, ~~thereby~~ in order to select excellent clones. In addition, clones obtained can be confirmed with stability of their ~~property, by repeating freezing~~ properties through repeated frozen storage.--

Please replace the paragraph from page 13, line 34, to page 14, line 9, with the following paragraph:

-- The term "stably expressing human cytochromes P450" is used to mean that the expression of human cytochromes P450 is not transient and specifically, the activity of cytochromes P450 is not lost when cells are cultured (subcultured). The cells capable of expressing human cytochromes P450 are preferably cells in which not only cytochromes P450 but also enzymes associated with various ~~metabolisms~~ aspects of metabolism (specifically, UDP-glucuronosyltransferase, sulfotransferase, glutathione transferase, epoxy hydratase, N-acetyltransferase, flavin monooxygenase, etc.) are capable of functioning.--

Please replace the paragraph at page 15, lines 1-17, with the following paragraph:

--For reproducing the function of the liver by expressing cytochromes P450, the cells should thus be those capable of functioning at least, human-derived UDP-glucuronosyltransferase, sulfotransferase, glutathione transferase, epoxy hydratase, N-acetyltransferase or flavin monooxygenase in the cells. One of such cells is cultured cell HepG2 originating from human hepatic carcinoma. The HepG2 cell is known to be capable of functioning UDP-glucuronosyltransferase, sulfotransferase, glutathione transferase, epoxy hydratase, N-acetyltransferase, flavin monooxygenase and NADPH P450 reductase function in HepG2 (J. Rueff et al., Mutation Research, 353, 151-176 (1996). In light of the foregoing, the present inventors have succeeded in stably expressing cytochromes P450 in HepG2 ~~thereby~~ in order to reproduce the function of human liver in a rapid, inexpensive, safe and accurate fashion.--

Please replace the paragraph at page 15, lines 18-21, with the following paragraph:

--In particular, ~~preferably used are~~ preferred embodiments include Hepc/3A4.5, Hepc/2E1.3-8, Hepc/2C9.1, Hepc/2C8.46, Hepc/1A2.9, Hepc/1A1.4, Hepc/2B6.68, Hepc/2D6.39, Hepc/2A6L.9, Hepc/2C19.12, etc.--

Please replace the paragraph from page 16, line 25, to page 17, line 10, with the following paragraph:

-- By analyzing a change in the structure of xenobiotics and/or endogenous substrates through exposure of a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450, enzymes that participate in the metabolism of xenobiotics and/or endogenous substrates can be analyzed (J. L. Napoli et al., Methods in Enzymology, vol. 206, pp. 491-501, Ed. by M. R. Waterman et al., Academic Press, 1991; H. K. Kroemer et al., Methods in Enzymology, vol. 272, pp. 99-108, Ed. by M. R. Waterman et al., Academic Press, 1996). Specific examples include identification of an enzyme participating the metabolism of xenobiotics and/or endogenous substrates by analyzing a change in the structure of xenobiotics and/or endogenous substrates upon exposure of a test specimen to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450~~,~~. Other specific examples include analysis of the

mechanism in an enzymatic reaction by analyzing a change in the structure of xenobiotics and/or endogenous substrates upon exposure of a test specimen of interest to the cell, and analysis of substrate specificity.--

Please replace the paragraph at page 18, lines 11-20, with the following paragraph:

-- By collecting the altered product (the so-called metabolite) from xenobiotics and/or endogenous substrates produced as a result of exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450 and purifying and ~~separating~~ isolating the product in an appropriate manner, the metabolite of xenobiotics and/or endogenous substrates can be prepared (J. L. Napoli et al., Methods in Enzymology, vol. 206, pp. 491-501, Ed. by M. R. Waterman et al., Academic Press, 1991).--

Please replace the paragraph from page 18, line 25, to page 19, line 4, with the following paragraph:

-- By exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450, the inhibition of a metabolizing enzyme for xenobiotics and/or endogenous substrates can be analyzed (J. L. Napoli et al., Methods in Enzymology, vol. 206, pp. 491-501, Ed. by M. R. Waterman et al., Academic Press, 1991). Specifically, the inhibition can be detected by the inhibition of cytochromes P450 enzyme activity, reduction in the amount of protein, decreased mRNA, etc. The detection may be made using publicly known methods, including an assay for enzyme activity corresponding to the respective members of P450, ~~western~~ Western blotting corresponding to the respective P450 proteins, ~~northern~~ Northern hybridization corresponding to various P450 mRNAs, RT-PCR, etc.--

Please replace the paragraph at page 19, lines 10-25, with the following paragraph:

-- By exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450 and detecting the increased enzyme activity in the metabolism of xenobiotics and/or endogenous substrates, the increased amount of the enzyme or the increased amount of transcription in gene encoding the enzyme, the

accelerated activity of the metabolizing enzyme for xenobiotics and/or endogenous substrates can be analyzed (J. Rueff et al., Mutation Research, 353 (1996) 151-176). Specifically, the accelerated activity can be analyzed by detecting the increased enzyme activity of cytochromes P450, the increased amount of protein or the increased mRNA. The detection may be made using publicly known methods, including ~~western~~ Western blotting corresponding to the respective P450 proteins, ~~northern~~ Northern hybridization corresponding to various P450 mRNAs, RT-PCR, etc.--

Please replace the paragraph from page 19, line 30, to page 20, line 8, with the following paragraph:

-- By exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450, the cytotoxicity caused by the metabolism of xenobiotics and/or endogenous substrates can be analyzed. Specifically, the cytotoxicity can be analyzed by observing a morphological change of the cell caused upon exposure of a test specimen~~,~~; a change in viable cell count determined by publicly known methods including the MTT assay, Trypan Blue staining, Crystal Blue staining, etc.~~,~~; leakage of intracellular enzyme such as lactose dehydrogenase~~,~~; a change in structure of cells in the top layer~~,~~; a change in intracellular enzyme, etc. (D. Wu, et al., Journal of Biological Chemistry, 271 (1996), 23914-23919).--

Please replace the paragraph at page 20, lines 13-28, with the following paragraph:

-- By exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450 and then subjecting the cells to the chromosomal aberration test or the micronucleus test, the genotoxicity caused by the metabolism of xenobiotics and/or endogenous substrates can be analyzed. The genotoxicity can also be analyzed by exposing a test specimen to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450 and then subjecting the cells to the chromosomal aberration test, to the micronucleus test or to the reverse mutation test~~,~~. This ~~which~~ involves assessment of the test specimen altered by the cells in an appropriate assessment system (J. Rueff et al., Mutation Research, 353 (1996) 151-176; M. E. McManus

et al., Methods in Enzymology, vol. 206, pp. 501-508, Ed. by M. R. Waterman et al., Academic Press, 1991).--

Please replace the paragraph from page 20, line 33, to page 21, line 12, with the following paragraph:

-- By exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450 and then subjecting the cells to the chromosomal aberration test or to DNA modification, the carcinogenicity caused by the metabolism of xenobiotics and/or endogenous substrates can be analyzed. The carcinogenicity can also be analyzed by exposing a test specimen to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450, followed by assessment of the test specimen altered by the cells in an appropriate system for evaluating carcinogenesis (J. Rueff et al., Mutation Research, 353 (1996) 151-176; K. Kawajiri, et al., Cytochromes, P450, Metabolic and Toxicological Aspects, pp. 77-98, ed. by C. Ioannides, CRC Press (1996)).--

Please replace the paragraph from page 21, line 35, to page 22, line 11, with the following paragraph:

-- The hepatotoxicity by the metabolism of xenobiotics and/or endogenous substrates can be analyzed ~~either~~ by exposing a test specimen, e.g., to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450 and then observing the expression of cytotoxicity~~, or~~. Alternatively, it can be analyzed by exposing a test specimen to the human hepatic carcinoma-derived cultured cell line capable of stably expressing cytochromes P450, administering the test specimen altered by the cells to other liver cells, liver slices or removed liver or to an experimental animal and then observing a change in cell or tissue or an in vivo change caused thereby.--

Please replace the paragraph at page 35, lines 11-30, with the following paragraph:

--HepG2 was maintained in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% FCS (fetal calf serum; Bio Whittaker). HepG2 was inoculated on a

60 mm dish and grown 50-60% confluently in a CO_2 incubator followed by transfection of 2 μg of 1A1/pcDNA3.1(+), 1A2/pcDNA3.1(+), 2A6/pcDNA3.1(+), 2B6/pcDNA3.1(+), 2C8/pcDNA3.1(+), 2C9/pcDNA3.1(+), 2C19/pcDNA3.1(+), 2D6/pcDNA3.1(+), 2E1/pcDNA3.1(+) or 3A4/pcDNA3.1(+) using lipofectamine reagent (GIBCO BRL). After incubating in 10% FCS-supplemented DMEM medium for 2 days, ~~a~~ the medium was replaced with fresh DMEM medium supplemented with 500 μg/ml G418 (GIBCO BRL) and 10% FCS. ~~was replaced for the medium. A fresh~~ The medium was replaced every 3 or 4 days to effect cloning of G418-resistant strains. The resulting G418-resistant strains were maintained in DMEM medium supplemented with 200 μg/ml G418 (GIBCO BRL) and 10% FCS. Each of the G418-resistant strains was assayed for the activity of cytochromes P450 by the method described below. Cell lines showing a high activity were measured and cells that expressed the high activity were selected.--

Please replace the paragraph at page 36, lines 3-18, with the following paragraph:

--CYP1A1- or CYP1A2-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, ethoxyresorufin previously diluted to 500 μM was added in 500 μl/well. After reacting at 37°C in the dark, the reaction solution was recovered from each well. After 1800 μl of methanol (Wako Junyaku K.K.) was added to 300 μl of the reaction solution and insoluble ~~matters were~~ material was removed by centrifugation, fluorescent intensity was measured at an excited wavelength of 550 nm and a fluorescence wavelength of 586 nm using a spectrofluorometer to quantify the resorufin formed. The product purchased from Molecular Probes was used as the standard substance for resorufin (Molecular Probes).--

Please replace the paragraph from page 36, line 30, to page 37, line 8, with the following paragraph:

-- CYP2A6-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium.

Subsequently, coumarin previously diluted to 500 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. The reaction solution was diluted to 10-fold with 0.1M Tris-HCl (pH 7.4) and fluorescent intensity was measured at an excited wavelength of 390 nm and a fluorescence wavelength of 440 nm using a spectrofluorometer to quantify 7-hydroxycoumarin formed. The product purchased from Extrasynthese was used as the standard substance for 7-hydroxycoumarin.--

Please replace the paragraph at page 37, lines 19-33, with the following paragraph:

-- CYP2B6-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, 7-ethoxycoumarin previously diluted to 500 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. The reaction solution was diluted to 10-fold with 0.1M Tris-HCl (pH 7.4) and fluorescent intensity was measured at an excited wavelength of 390 nm and a fluorescence wavelength of 440 nm, using a spectrofluorometer (Hitatchi Spectrofluorometer F-2000) to quantify 7-hydroxycoumarin formed. The product purchased from Extrasynthese was used as the standard substance for 7-hydroxycoumarin.--

Please replace the paragraph at page 38, lines 9-20, with the following paragraph:

-- CYP2C8-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, taxol diluted previously to 30 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. After an equal volume of acetonitrile (Wako Junyaku K.K.) was added to and mixed with the reaction solution, insoluble ~~matters were~~ material was removed by centrifugation. 6α-Hydroxypaclitaxel formed in the reaction solution was quantified on HPLC.--

Please replace the paragraph at page 39, lines 6-17, with the following paragraph:

-- CYP2C9-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, tolbutamide previously diluted to 500 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. After an equal volume of acetonitrile (Wako Junyaku K.K.) was added to and mixed with the reaction solution, insoluble ~~matters were~~ material was removed by centrifugation. Hydroxytolbutamide formed in the reaction solution was quantified on HPLC.--

Please replace the paragraph at page 40, lines 4-15, with the following paragraph:

-- CYP2C19-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, mephenytoin previously diluted to 100 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. After an equal volume of acetonitrile (Wako Junyaku K.K.) was added to and mixed with the reaction solution, insoluble ~~matters were~~ material was removed by centrifugation. 4'-Hydroxymephenytoin formed in the reaction solution was quantified on HPLC.--

Please replace the paragraph at page 41, lines 2-10, with the following paragraph:

-- CYP2D6-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, bufralol previously diluted to 200 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well and 1'-hydroxybufralol formed in the reaction solution was quantified on HPLC.--

Please replace the paragraph from page 41, line 34, to page 42, line 11, with the following paragraph:

--2E1-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a

CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, p-nitrophenol previously diluted to 500 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. To 100 μl of the reaction solution 50 μl of NaOH (Wako Junyaku K.K.) was added, and insoluble ~~matters were~~ material was removed by centrifugation. By measuring absorbance at 540 nm - 620 nm, 4-nitrocatechol formed was quantified. 4-Nitrocatechol purchased from Wako Junyaku K.K. was used as the standard substance.--

Please replace the paragraph at page 42, lines 22-33, with the following paragraph:

-- CYP3A4-expressing cells were inoculated on a 12-well plate (Falcon) and incubated in a CO_2 incubator so as to become confluent. After incubation, the medium was suctioned and cells ~~adhered~~ adhering to the plate were washed with Phenol Red-free DMEM medium. Subsequently, testosterone previously diluted to 100 μM was added in 500 μl/well. After reacting at 37°C, the reaction solution was recovered from each well. After an equal volume of acetonitrile (Wako Junyaku K.K.) was added to and mixed with the reaction solution, insoluble ~~matters were~~ material was removed by centrifugation. 6β-Hydroxytestosterone formed in the reaction solution was quantified on HPLC.--

Please replace the paragraph at page 46, line 8, with the following paragraph:

--~~Reults:~~ Results: see FIGS. 1 and 2--

Please replace the paragraph at page 49, line 10, with the following paragraph:

--~~Reults:~~ Results: see FIGS. 4 and 5--

Please replace the paragraph at page 49, lines 12-27, with the following paragraph:

-- It is considered that after cyclophosphamide is hydrolyzed at the 4-position, phosphoramide or acrolein formed by non-enzymatic degradation would act as an alkylating agent to cause hepatic cytotoxicity triggered by covalent binding to macromolecular components in liver cells (K. H. Thomas, et al., Cancer Research, vol. 53, pages 5629-5637,

1993). Cyclophosphamide caused a leakage of LDH in Hepc/2B6.68 cells concentration-dependently in the concentration up to 2 mM and then reached the plateau at the following concentrations. Cyclophosphamide was slightly cytotoxic also in HepG2 (FIG. 4). Turning to the MTT assay method, cyclophosphamide caused a slight decrease of the MTT activity concentration-dependently in Hepc/2B6.68 cells (FIG. 5). ~~Cyclphosphamide~~ Cyclophosphamide was metabolized by the CYP2B6 activity, and the metabolic intermediate formed showed cytotoxicity.--

REVISIONS OF CLAIMS 1-4 PURSUANT TO REVISED RULE § 1.121

Pursuant to Revised Rule § 1.121(c)(1)(ii), the revisions of claims 1-4 are detailed as follows:

In the Claims:

--1 (Amended). ~~A~~ An isolated cell line derived from a human ~~hepatic carcinoma capable of stably expressing human cytochromes P450.~~ hepatocarcinoma cell, which stably expresses human cytochrome P450, which is introduced by transfection, provided that when the human hepatocarcinoma cell is HepG2, the human cytochrome P450 is other than CYP2E1.

2 (Amended). The cell line according to claim 1, ~~wherein human cytochromes P450 are capable of stably expressing~~ which stably expresses CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, ~~CYP2E1~~ or CYP3A4.

3 (Amended). The ~~cultured~~ cell line according to claim 1, wherein the human ~~hepatic carcinoma~~ hepatocarcinoma cell is HepG2.

4 (Amended). The cell line according to claim 1, which is Hepc/1A1.4, Hepc/1A2.9, Hepc/2B6.68, Hepc/2C8.46, Hepc/2C9.1, Hepc/2C19.12, Hepc/2D6.39, ~~Hepc/2E1.3-8~~ or Hepc/3A4.5.--

REVISIONS OF THE ABSTRACT PURSUANT TO REVISED RULE § 1.121

In the Abstract:

Please replace the abstract (two paragraphs) at page 57, lines 3-11, with the following new abstract (one paragraph):

--This invention relates to cell lines that are obtained using cultured cell lines derived from human liver as a host and that stably express a number of human cytochromes P450. The human liver-derived cultured cell lines of the present invention are useful in, for example, analyzing an enzyme participating in the metabolism of xenobiotics or endogenous substrates, because of their stable expression of human cytochromes P450 CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, ~~2E1~~ and 3A4.--